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13. ABSTRACT *(Maximum 200 words)*

The goal of this research program was to enhance our understanding of the molecular mechanisms that underlie odor recognition. Odorant receptors are G-protein-linked receptors that couple binding of odorants to activation of adenylyl cyclase or phospholipase C. They show extensive sequence diversity in their transmembrane domains, especially the fourth and fifth transmembrane helices, referred to as the "hypervariable odorant binding domain." A chimeric receptor was constructed in which a cassette encoding the hypervariable odorant binding domain of rat odorant receptor I-15 was inserted in the human β_2-adrenergic receptor. This construct was cloned in a eukaryotic expression vector and expressed in stably transfected cells. This system forms the basis for future structure/function studies on the interactions between odorants and this hypervariable odorant binding domain. We initiated two additional lines of research that can provide insights in odor recognition and its relation to odor-guided behavior. One approach utilizes avoidance behavior toward certain odorants displayed by *Drosophila melanogaster*. This organism is amenable to genetic, molecular biological, neuroanatomical and behavioral studies, allowing multidisciplinary studies. The other approach centers on recognition of pheromones that trigger reproductive behavior via the vomeronasal organ and characterization of the signal transduction pathways they activate.

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1. FOREWORD

This final progress report describes results obtained with support from ARO grant DAAH04-94-G-0027. ARO grant DAAH04-94-G-0027 continued the research program of DAAL03-92-G-0390 and was activated when the Principal Investigator moved from Duke University to North Carolina State University. The present report covers the period from March 15, 1994 through December 14, 1995. It describes studies on the functional characterization of odorant receptors that set the stage for experiments proposed in proposal 34815-LS, currently under consideration by the ARO. In addition, thanks to funds provided by the ARO under DAAH04-94-G-0027, we were able to diversify our strategies toward understanding the molecular basis of odor recognition by initiating two additional lines of research that have been highly successful. The results of these studies are also included in this progress report and describe the identification of novel olfactory genes in *Drosophila melanogaster* and elucidation of a signal recognition and transduction pathway for mammalian pheromones. Both of these additional lines of research, aimed at gaining insights in odor recognition in behaviorally well characterized systems, grew into independent comprehensive research programs, which are now too large to be supported by future ARO support alone. One of these projects (Molecular genetics of olfaction in Drosophila), therefore, is now supported by a grant from the NIH (RO1-DC02485) and grant applications to support the second project (Chemoreception of mammalian pheromones) have been submitted to the North Carolina Biotechnology Center and the NIH. ARO proposal 34815-LS, when funded, will continue the central line of research originally described in DAAH04-94-G-0027, i.e. *in vitro* characterization of odorant receptors.

2. TABLE OF CONTENTS

3. LIST OF APPENDICES, ILLUSTRATIONS AND TABLES 4

4. BODY OF REPORT 5

A. STATEMENT OF THE PROBLEM STUDIED 5

B. SUMMARY OF THE MOST IMPORTANT RESULTS 6

1. Construction of a chimaeric odorant receptor-β_2-adrenergic receptor 6

2. Molecular genetics of olfaction in *Drosophila melanogaster* 9

3. Pheromone recognition and signal transduction in the mammalian vomeronasal organ 11

C. LIST OF ALL PUBLICATIONS AND TECHNICAL REPORTS 14

D. LIST OF ALL PARTICIPATING SCIENTIFIC PERSONNEL 16

5. REPORT OF INVENTIONS 16

6. BIBLIOGRAPHY 17

7. APPENDICES 22

3. LIST OF APPENDICES, ILLUSTRATIONS AND TABLES

Figure 1: Activation of adenylyl cyclase in control Hela cells and transfected HeLa cells expressing a chimeric β_2-adrenergic receptor containing the hypervariable odorant binding domain of rat odorant receptor I-15 by pools of odorants.

Figure 2: Generation of inositol-(1,4,5)-triphosphate by boar urine in microvillar membranes from vomeronasal organs of 3 month-old prepubertal gilts.

Table 1: Odorants used for initial screening assays.

Table 2: *P[lArB]* insert lines of *Drosophila melanogaster* with aberrant olfactory behavior.

Table 3: Generation of cyclic AMP and inositol-(1,4,5)-triphosphate in microvillar membrane preparations from female porcine vomeronasal organs

4. BODY OF REPORT

A. STATEMENT OF THE PROBLEM STUDIED

Virtually all animals depend on chemoreception for survival. The repertoire of biologically relevant chemical signals can be classified into two broad classes: signals that provide information regarding the presence of food, predators and environmental toxins; and, signals that convey social information regulating interactions between conspecifics, such as parental recognition and maternal behavior, selection of reproductive partners and mating behavior, and establishment of dominance patterns and territorial boundaries. Most vertebrates have developed a dual chemosensory system for separate recognition and information processing of each of these classes of signals, the main olfactory system and the vomeronasal (accessory olfactory) system, respectively.

In the main olfactory system, odor recognition is triggered by binding of odorants to odorant receptors on the dendritic cilia of olfactory receptor neurons (reviewed by Anholt, 1993). This interaction activates G-protein-mediated generation of cyclic AMP (Pace *et al.*, 1985; Sklar *et al.*, 1986; Shirley *et al.*, 1986; Anholt *et al.*, 1987; Jones and Reed, 1989; Bakalyar and Reed, 1990; Breer *et al.*, 1990). The subsequent opening of cyclic nucleotide-activated channels then allows an influx of cations that carry the generator current (Nakamura and Gold, 1987; Dhallan *et al.*, 1990; Ludwig *et al.*, 1990; Firestein *et al.*, 1991; Frings and Lindemann, 1991; Gouldin *et al.*, 1992). Inositol triphosphate has been implicated as an alternative second messenger, but the mechanism by which it controls neuronal excitation is less well characterized (Huque and Bruch, 1986; Boekhoff *et al.*, 1990; Breer *et al.*, 1990; Breer and Boekhoff, 1991; Ronnett *et al.*, 1993). Olfactory transduction is regulated by calcium entering through cyclic AMP-activated channels. Calcium enhances the response to odorants by activating a calcium-dependent chloride current (Kleene and Gesteland, 1991; Kurahashi and Yau, 1993; Lowe and Gold, 1993) and, as a calcium-calmodulin complex, by stimulating adenylyl cyclase (Anholt and Rivers, 1990). In addition, calcium-calmodulin limits the duration of the response by interacting directly with the cyclic nucleotide-activated channel and decreasing its affinity for cyclic AMP (Chen and Yau, 1994; Liu *et al.*, 1994). Prolonged exposure to odorants leads to desensitization via phosphorylation by protein kinase A, protein kinase C (Boekhoff and Breer, 1992) and GRK-2 (Dawson *et al.*, 1993; Schleicher *et al.*, 1993; Boekhoff *et al.*, 1994). The latter specifically phosphorylates liganded odorant receptors (Dawson *et al.*, 1993; Schleicher *et al.*, 1993; Boekhoff *et al.*, 1994).

Populations of olfactory neurons expressing different odorant receptors generate distinct patterns of neural activity, that encode the quality and concentration of odorants (reviewed by Anholt, 1993). Decoding of the odor message is achieved through sorting of cells with similar or overlapping odorant receptor specificities into an organized neural projection pattern. Stochastic processes of allelic inactivation and linking of *cis* promoter elements to a particular odorant receptor gene restrict the expression of odorant receptor genes to one or few per olfactory neuron (Chess *et al.*, 1994). Furthermore, olfactory neurons with the same odorant specificity converge on the same mitral cell in the olfactory bulb (Vassar *et al.*, 1994). Thus, dispersion of cells with similar odorant specificities along the epithelial sheet and their convergence to the same glomerulus in the bulb increases the probability for odorant detection

as air flows over the olfactory epithelial sheet. Olfactory discrimination, however, ultimately depends on ligand specificities of individual odorant receptors.

Odorant receptors comprise a large multigene family that belongs to the superfamily of G-protein-linked heptahelical receptors (Buck and Axel, 1991; Levy *et al.*, 1991; Selbie *et al.*, 1992; Ngai *et al.*, 1993; Ben-Arie *et al.*, 1993; Raming *et al.*, 1993). In addition to the familiar α-helical seven transmembrane motif they contain conserved sequence motifs characteristic of the odorant receptor subfamily. Compared to most heptahelical receptors, odorant receptors have short intracellular and extracellular loops and a short extracellular N-terminal domain. The extracellular loops contain an unusually large proportion of hydrophobic amino acid residues. The most distinguishing feature of members of the odorant receptor family, however, is the pronounced sequence diversity in the transmembrane domains, especially the fourth and fifth transmembrane helices that constitute "the hypervariable odorant binding domain" (reviewed by Lancet and Ben-Arie, 1993). Sequence diversity in these transmembrane regions is thought to reflect structural diversity of the ligand binding site. Thus, the transmembrane core of the receptor appears instrumental in determining specificity of the odorant binding site. The primary goal of the previous research period was to characterize how the hypervariable domain of the ligand binding site of an odorant receptor interacts with odorants by measuring odorant-stimulated adenylate cyclase in eukaryotic cells that express a chimeric odorant receptor-β_2-adrenergic receptor construct.

During the course of our studies we had the opportunity to initiate two additional lines of research that can provide insights in the molecular basis of odor recognition. These make use of experimental systems in which odors induce distinct behaviors, thus allowing us to place odor recognition in a physiologically relevant behavioral context. One of these systems is the avoidance behavior toward certain odorants displayed by *Drosophila melanogaster*. This organism has the great advantage that it allows a multidisciplinary approach using well-characterized genetics, avant-garde molecular biology, neuroanatomical and behavioral studies all in the same organism. The second system is the recognition of mammalian pheromones that trigger reproductive behavior via the vomeronasal organ. Below, we will first describe the construction of a chimaeric odorant receptor-β_2-adrenergic receptor, which can be used to study structure/function relationships within the hypervariable odorant binding domain. This will be followed by an account of our studies on the molecular genetics of olfaction in *Drosophila melanogaster* and vomeronasal chemoreception of mammalian pheromones.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

1. Construction of a chimaeric odorant receptor-β_2-adrenergic receptor

We constructed a cDNA that encodes a chimaeric receptor consisting of the β_2-adrenergic receptor (Kobilka *et al.*, 1987) of which the fourth and fifth transmembrane domains and their extracellular linker were replaced by a cassette that corresponds to the equivalent region of odorant receptor I-15 (Buck and Axel, 1991). The clone encoding the rat odorant receptor I-15 in the pBluescript vector was generously donated by Dr. Linda Buck and a clone encoding the human β_2-adrenergic receptor, also in pBluescript, was donated by Dr. Robert J. Lefkowitz. The region of the odorant receptor that encodes the fourth and fifth transmembrane domains was

amplified by PCR using the following 25-mer primers: 5'-TGATCAGTCTGGTGGTGCT-GTCCTG-3' and 5'-GTAGAC-AATGATGAGCACAATGGA-3'. The resulting amplification product of approximately 225 bp contains restriction sites for Bcl1 and Acc1. This PCR product was cloned into the T-A cloning vector of Invitrogen (La Jolla, CA) and can be released from the vector by double digestion with Bcl1 and Acc1. The β_2-adrenergic receptor clone contains restriction sites for Bcl1 at nucleotide positions 1718 and 1901 and an Acc1 restriction site at nucleotide 1916. Thus a 192 bp fragment can be excised from this cDNA that encodes the fourth and fifth transmembrane domains of this receptor and exchange of this region by the Bcl1/Acc1 digested odorant receptor PCR fragment described above would provide an intact open reading frame that encodes the desired chimaeric receptor. In order to be able to make this construct we first had to subclone the cDNA encoding the β_2-adrenergic receptor into pGEM7Zf(+), a vector that contains neither a Bcl1 nor an Acc1 restriction site. The cDNA that encodes the β_2-adrenergic receptor subcloned into pGEM7Zf(+) was digested with Bcl1 and Acc1 and the Bcl1/Acc1 released insert from the T-A cloning vector containing the PCR fragment that encodes the odorant binding domain was ligated into the linearized pGEM7Zf(+) construct. To verify that the chimaera was formed correctly in frame the regions spanning both ligation sites were sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The corresponding amino acid sequences of the original β_2-adrenergic receptor in these regions are:

...LLTKNARVI---ILMVW.....PLVIMVFV---YSRVFQ...

(--- indicates the positions of ligation sites)

The corresponding amino acid sequences of odorant receptor I-15 in these regions are as follows:

...IMSPKLCV---SLVVS.....PFVLIIVS---YARVVA...

The cDNA for the chimaeric receptor which we constructed encodes the amino acid sequences:

...LLTKNKARVI---*SLVVS*.....*PFVLIIV*---YSRVFQ....

To functionally characterize the chimeric receptor encoded by our cDNA construct we subcloned the construct into the eukaryotic expression vector pCEP4. This is a 10.4 kb Epstein Barr virus derived vector that in primate cells replicates extrachromosomally to high copy number (app. 30/cell). pCEP4 carries the β-lactamase gene that confers ampicillin resistance for maintenance in E. coli. We cloned our chimeric cDNA construct and the cDNA encoding the parental β_2-adrenergic receptor between the Xho1 and BamH1 sites of the multiple cloning site in the correct orientation to allow expression of the chimeric receptor under the control of a strong cytomegalovirus promoter. We established optimal conditions for transfection of HeLa cells via a calcium phosphate precipitation procedure. Since the pCEP4 vector confers hygromycin B resistance to the cells, we grow transfectants in the presence of 200 μg/ml hygromycin B to select and maintain stable transfectants. The first transfectants we obtained failed to express the receptors encoded by the cDNAs cloned in pCEP4, because the 5'-untranslated region placed the ATG initiation codons too far from the CMV promoters. We designed two strategies to bring the transcription initiation sites closer to the CMV promoters. First we linearized the constructs by restriction digestion with Xho1 followed by bidirectional digestion with exonuclease BAL31 to remove nucleotides from the 5' untranslated regions and

the multiple cloning sites near the CMV promoters. Restriction analysis with BglII and KpnI was used to ascertain that the exonuclease left the CMV promoter sequences and the open reading frames of the inserts intact. Recircularization following BAL31 digestion would then lead to constructs in which the ATG initiation transcription codons would be in closer proximity to the CMV promoters. However, we were not able to control the exonuclease reaction with sufficient accuracy and generated either overdigested or underdigested products. We then decided to adopt an alternative strategy, namely to amplify by PCR the open reading frames of the cDNAs encoding the chimeric receptor and the parental β_2-adrenergic receptor using primers that would generate an Xho1 site and a BamH1 site in the amplification product. The forward primer was 5'AAAAAACTCGAGTTACCTGCCAGACTGCGCGCCATGGGGCAA3' (the Xho1 restriction site is indicated in italics and the initiation codon is underlined) and the reverse primer was 5'GGGGGG*GGATCC*TTACAGCAGTGAGTCATTTGTACTACAATT3' (the BamH1 restriction site is indicated in italics). The amplified fragments were analyzed by agarose gel electrophoresis and corresponded in sizes to the open reading frames of the cDNA inserts. They were purified and ligated in Xho1/BamH1-digested pCEP4 placing the initiation codons in close proximity to the CMV promoters.

We have obtained stable HeLa cell transfectants that express the chimeric receptor, as evident from Northern blot analysis, and we have started to screen systematically sets of odorants for activation of the chimeric receptor. We are using for our initial screens three sets of odorants (Table 1): (1) A set of esters that vary systematically in chain length, branching, unsaturation and ester moiety, provided as an organoleptic test kit by Aldrich Chemical Company (Milwaukee, WI; catalogue number W60000-8); (2) A similar set of heterocyclics, also provided as a systematic test kit by Aldrich Chemical Company (catalogue number W60002-4); and, (3) A set of standard odorants, that are widely used and donated by International Flavors and Fragrances, Inc. (Union Beach, NJ). Initially, we screen our odorants as pools. In the case of the Aldrich test kits, rows and columns of the kit, consisting of 6 and 4 odorants, respectively, are pooled, so that each odorant occurs in two different pools. In this way 24 molecules can be screened by assaying only 10 pools. An initial screen using the Aldrich ester kit identified two pools of odorants that showed activation of adenylate cyclase to 77% and 70%, respectively, of activation by forskolin (Fig. 1). The common odorant represented in each of these two pools is furfurylpropionate, an ester with a spicy, floral aroma. Although these results are very encouraging, it should be noted that they are preliminary and need to be consolidated by characterizing the dose-response behavior and GTP-dependence, and by assaying a number of structurally similar furfuryl esters individually to rule out possible artifacts. These studies are currently in progress and, hopefully, can be continued, when our pending ARO continuation proposal 34815-LS is funded.

2. Molecular genetics of olfaction in *Drosophila melanogaster*

We postulate that detection of an odorant, such as benzaldehyde, involves interactions between the odorant and multiple members of the odorant receptor family similar to the recognition of an antigen by a spectrum of antibodies in a polyclonal antiserum. Although considerable progress has been made recently in elucidating the molecular basis of odor recognition and olfactory transduction, a gap remains in our understanding of how the concerted expression of an ensemble of genes shapes odor-guided behavior. To approach this complex issue it is helpful to view olfaction as a standard genetic multifactorial trait. We, therefore, initiated a collaboration with Dr. Trudy Mackay of the Department of Genetics at North Carolina State University, a leading authority on quantitative genetics of *Drosophila melanogaster.*

Drosophila melanogaster has a relatively simple olfactory system consisting of approximately 1000 olfactory neurons in the third antennal segment and the maxillary palps (reviewed by Carlson, 1991, and Stocker, 1994). The simplicity of its olfactory system and the eminent suitability of *Drosophila* for genetic and molecular biological analyses make it an ideal model system for investigating the genetic basis of odor-guided behavior.

A simple and rapid quantitative assay was developed to assay olfactory behavior in *Drosophila* and to detect lines with aberrant olfactory behavior. Using single sex groups of five flies we measured at ten 5-second intervals the number of flies that moved into a compartment remote from an odor source. We calculated the average number of flies in this compartment per time point as the "avoidance score". A score of 2.5 indicates the flies are indifferent to the chemosensory stimulus, spending as much time near its source as away from it; scores greater than 2.5 indicate they are repelled by the stimulus and scores less than 2.5 indicate the stimulus attracts them.

To determine standard genetic parameters that describe quantitative variation of odor-guided behavior in natural populations of *Drosophila melanogaster*, we substituted 43 *X* and 35 third chromosomes from a natural Raleigh population of *Drosophila melanogaster* into an isogenic Samarkand strain and the resulting chromosome substitution lines were subjected to our behavioral assay that quantitates avoidance responses to benzaldehyde. Phenotypic variation in olfactory responsiveness was determined for males and females separately and was similar for the *X* and third chromosome substitution lines. Analysis of gender correlations among lines showed that phenotypic variation among males had a different genetic basis from that among females, indicating that different alleles contribute differently to odor-guided behavior in each sex. Since variation for olfactory responsiveness was small compared to that for other multifactorial traits and since the poor correlation among sexes suggested that they interact differently with their chemosensory environment, the hypothesis was pursued that olfaction may be a fitness trait. Viability and fertility of males and females of each of the substitution lines were evaluated against a *Ubx*-marked competitor strain and correlated with olfactory avoidance scores. Whereas male fertility and viability of either sex were uncorrelated with odor-guided behavior, olfactory responsiveness showed a significant correlation with female fertility. These observations suggest that in *Drosophila melanogaster* the genome interacts with the chemosensory environment in a sex-dependent manner and that adaptive evolutionary pressures that restrict phenotypic variation in olfactory responsiveness act on female fertility.

To identify specific quantitative trait loci for olfaction, we introduced a marked *P*-element

construct (*P[lArB]*) into our highly inbred Samarkand strain. Random stable integration of a single *P*-element construct in the genome results in a library of co-isogenic lines. The *P[lArB]* construct prefers to insert in regulatory regions, thereby disrupting expression of genes adjacent to the insertion sites. A *lacZ* reporter gene contained in the *P[lArB]*-element allows us to confirm that the loci identified are expressed in olfactory tissues. In addition, *P[lArB]*-tagged loci can be mapped cytogenetically by *in situ* hybridization to larval polytene salivary chromosomes. Furthermore, the *P[lArB]*-element contains the pBluescript vector and, thus, tags these loci for cloning and subsequent characterization of their gene products.

We measured odor-guided behavior in 94 replicates of the isogenic *P*-element free control strain and in 379 lines in which *P[lArB]* elements had been introduced into the genome. The latter consisted of 188 second chromosome *P[lArB]* insert lines and 191 third chromosome *P[lArB]* insert lines. For the controls the mean avoidance score (± standard error) to water was 1.69 ± 0.03 and to benzaldehyde was 3.95 ± 0.03. For the *P[lArB]* insert lines, the population mean avoidance scores to water and benzaldehyde were 1.53 ± 0.06 and 3.59 ± 0.05, respectively, for the second chromosome lines; and 1.57 ± 0.03 and 3.73 ± 0.03, respectively, for the third chromosome lines. We identified 8 second and 6 third chromosome lines that showed a weak or delayed response. Four of these lines displayed sexual dimorphism with a statistically significant larger impairment in the female (Table 2), in line with the sexually dimorphic phenotypic variation observed for the chromosome substitution lines, described above.

To assess whether olfactory deficits were specific to benzaldehyde or reflected general hyposmia, we measured avoidance responses to 2-isobutylthiazole and 2-*n*-propylpyrazine, odorants that are structurally unrelated to benzaldehyde. Twelve of the lines displayed general hyposmia. Therefore, these loci probably do not encode odorant receptors, but may encode gene products contributing to a common olfactory pathway. Some specificity, however, was observed in two of the sexually dimorphic lines. In one line males showed normal olfactory behavior when tested with benzaldehyde and 2-isobutylthiazole, but were hyposmic with respect to 2-*n*-propylpyrazine. In contrast, flies of another line showed normal avoidance responses to 2-*n*-propylpyrazine, but females responded poorly to 2-isobutylthiazole and low concentrations of benzaldehyde.

In lines in which expression of the *lacZ* reporter gene was observed β-galactosidase activity was evident in olfactory organs (the second and third antennal segments and/or maxillary palps). Whereas in most lines staining in the third antennal segment was uniform, regional expression in restricted areas of the third antennal segment was found in some of the lines. Expression of at least 10 of our transposon-tagged genes is controlled by olfactory tissue-specific promoter/enhancer elements.

Sites of *P*-element insertion were mapped by *in situ* hybridization to larval polytene salivary chromosomes. *P*-element insertions in different lines are at different locations (Table 2). The paucity of plausible candidate genes at these loci suggests that most, if not all, of them represent novel olfactory genes. These transposon-tagged genes can be isolated and are likely to encode a diverse ensemble of proteins essential for odor-guided behavior, such as proteins that mediate odor recognition and olfactory transduction, neural signal processing and integration of olfactory information, and transcription factors or extracellular matrix proteins that direct neural development. From the frequency of lines that show aberrant olfactory responses among our library of *P*-element insert lines, we estimate that at least 4% of the *Drosophila* genome

participates in mediating odor-guided behavior.

To rescue *P[lArB]*-tagged DNA sequences from our *smi* lines we digested genomic DNA with HindIII and recovered the *P*-element tagged sequences as inserts in pBluescript. *In situ* hybridization of these inserts to polytene chromosomes from the *P*-element free host strain and Southern blot analysis verified that they indeed derive from the original *P[lArB]* insertion sites. Whereas most rescued DNA fragments hybridize to a single chromosomal band, two *smi* sequences revealed additional hybridization sites. *P[lArB]*-tagged DNA from line *smi97B*, which shows major olfactory impairment and reporter gene expression in a restricted region of the antenna, hybridizes to several chromosomal bands in addition to band 97B, indicating that it represents a gene family. Similarly, Southern blots indicate that the gene tagged by the *P*-element in line *smi98B* may also be a member of a gene family. DNA rescued from line *smi79E* hybridizes extensively to heterochromatin in addition to multiple euchromatic bands, suggesting that the *P[lArB]*-construct may have inserted in or near a *hoppel* transposon. *P[lArB]*-tagged DNA fragments rescued from the *smi* lines are currently used to identify and characterize mRNA transcripts from a *Drosophila melanogaster* head cDNA library. Finally, reversion of the *smell impaired* phenotype through excision of the *P*-element will provide ultimate proof that aberrant olfactory phenotypes indeed result from *P*-element insertions and Northern blots will confirm that *smi* messages are reduced in *smi* lines when compared to the P-element free host strain.

The initiation of this exciting research program was made possible by funding from ARO grant DAAH04-94-G-0027. Since this project rapidly grew beyond the scope of DAAH04-94-G-0027, we established it as an independent research program and were able to obtain funding for its continuation from the NIH under grant RO1-DC02485.

3. Pheromone recognition and signal transduction in the mammalian vomeronasal organ

In contrast to the main olfactory system, little is known about molecular mechanisms of chemoreception in the vomeronasal organ. In many animals, pheromones released by conspecifics trigger reproductive, maternal and aggressive behaviors (Fleming *et al.*, 1979; Johnston, 1983; Clancy *et al.*, 1984; Coquelin *et al.*, 1984; Vandenbergh, 1994). Such pheromones are perceived via a distinct chemosensory organ, the vomeronasal organ, where they interact with dendritic microvilli of chemosensory neurons (Powers and Winans, 1975; Wysocki *et al.*, 1982; Halpern, 1987; Meredith, 1983 and 1991; Wysocki and Meredith, 1987; Stern, 1990). Recently, Dulac and Axel (1995) identified a family of putative pheromone receptors expressed in the VNO. The ligand specificities of these putative pheromone receptors, however, have not been characterized, nor have the transduction pathways to which they would be coupled been identified.

The vomeronasal organs are located bilaterally above the roof of the palate and adjacent to the septum embedded in the vomeronasal bone. They consist of cigar-shaped blind sacs of tissue from which ducts lead to the oral (most mammals, including pigs) or nasal (horses, lagomorphs) cavity (Meredith, 1983; Wysocki and Meredith, 1987). The organ contains vascular erectile tissue that can pump liquids into a lumen (Meredith and O'Connell, 1979; Eccles, 1982) which is lined by a pseudostratified sensory epithelium that contains vomeronasal neurons. These neurons are bipolar; they contain dendrites that at their apical ends sprout microvilli protruding into the lumen of the vomeronasal organ and axons that form the vomeronasal nerve (see Wysocki and Meredith, 1987). The vomeronasal nerve projects along the nasal septum through

the cribriform plate of the ethmoid bone to the accessory olfactory bulb. From there output neurons travel primarily to nuclei of the amygdala that in turn send projections to nuclei in the basal forebrain, including the bed nucleus of the stria terminalis and the ventromedial hypothalamus, that is intimately associated with reproductive behavior, through the release of GnRH (Scalia and Winans, 1975; Lehman and Winans, 1983).

One of the best documented pheromonal effects is the puberty acceleration effect (Vandenbergh, 1969 and 1994; Vandenbergh *et al.*, 1976; Lepri and Vandenbergh, 1986). When prepubertal female mice are exposed to an adult male they undergo accelerated onset of puberty accompanied by a marked increase in uterine weight (Vandenbergh, 1994; Brown, 1985). This male-induced acceleration of puberty is obliterated by lesions of the vomeronasal organ or the accessory olfactory bulb (Kaneko *et al.*, 1980). Puberty acceleration can be observed after a single application of a few drops of male urine to the oral groove of a juvenile female. It is mediated via a rapid pheromone-induced surge in GnRH that within two days results in a more than 10-fold increase in uterine weight (Bronson and Desjardins, 1974). This puberty enhancing effect is not unique to rodents, but has also been documented for pigs, whose reproductive behavior is strongly influenced by pheromones (Signoret *et al.*, 1975; Dorries, 1992). Gilts housed in the proximity of boars reach puberty earlier and this effect can, again, be attributed to boar urine (Pearce and Hughes, 1987).

Pigs present an ideal system for studies on vomeronasal chemoreception. Their sexual maturation and reproductive behavior are strongly influenced by pheromones (Signoret, 1970; Dorries, 1992). Their vomeronasal organs are large, well separated from the main olfactory system, and relatively easy to dissect. Furthermore, between the Animal Science Department's Swine Research Unit and the College of Veterinary Medicine at North Carolina State University we can collect large quantities of fresh porcine tissue.

To study the molecular mechanisms by which pheromones activate chemosensory neurons in the vomeronasal organ, we partially purified microvillar dendritic membranes from porcine vomeronasal organs. These microvillar membrane preparations contain 3.3% of total VNO membrane protein and are approximately 3-fold enriched in specific activity of forskolin-stimulated adenylate cyclase (Table 3). This adenylate cyclase activity, however, is approximately 50-fold lower than that measured previously in olfactory cilia preparations from frog (Pace *et al.*, 1985; Sklar *et al.*, 1986) or rat (Sklar *et al.*, 1986; Shirley *et al.*, 1986). In contrast to the observation that in snake VNOs prey-derived chemoattractants inhibit adenylate cyclase (Luo *et al.*, 1994), we did not detect either stimulation or inhibition of adenylate cyclase by several pheromonal stimuli, including the lordosis-inducing pheromone from boar saliva, 5α-androst-16-en-3-one (Patterson, 1968; Perry *et al.*, 1980) up to 10 μM, whole boar saliva (Perry *et al.*, 1980) or boar urine (Pearce and Hughes, 1987).

Microvillar membrane preparations also contain a G protein-coupled phospholipase C. GTPγS stimulates inositol-(1,4,5)-triphosphate production and this increase in inositol-(1,4,5)-triphosphate production is approximately 3-fold greater in microvillar fractions than in residual membrane fractions, paralleling the increase in specific activity of forskolin-stimulated adenylate cyclase (Table 3).

In three different vomeronasal membrane preparations from gilts, application of boar urine increased inositol-(1,4,5)-triphosphate production to the same levels as GTPγS (211 ± 34 pmol inositol-(1,4,5)-triphosphate/mg protein/min; Fig. 2). Boar urine contains, therefore, at least one

pheromone that activates phospholipase C in vomeronasal membranes from juvenile females. We did not detect activation of vomeronasal phospholipase C by 5α-androst-16-en-3-one up to concentrations of 10 μM or by boar saliva.

Western blotting with antibodies specific to G protein α subunits reveals that these membranes contain $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and a 40 kD polypeptide related to $G\alpha_q$. In contrast to $G\alpha_{i2}$ and $G\alpha_o$, previously localized to the cell bodies, axons and dendrites of subpopulations of vomeronasal neurons (Halpern *et al.*, 1995), the $G\alpha_q$-related protein is found mainly at the microvillar surface of the vomeronasal neuroepithelium. The large number of G protein α subunits associated with vomeronasal neurons implies multiple transduction pathways. The concentration of the $G\alpha_q$-related protein at the dendritic microvilli suggests a central role for this G protein in pheromone signalling.

Preliminary studies indicate that prolonged incubation with boar urine results in stimulus-dependent phosphorylation of a 44 kD protein, about the size predicted for a putative pheromone receptor (Dulac and Axel, 1995). A monoclonal antibody against GRK-2 and GRK-3 detects both of these G protein-coupled receptor kinases in the microvillar membrane preparation. Moreover, antiserum against GRK-2, like the anti-$G\alpha_q$ antiserum, stains the microvillar surface of the vomeronasal organ. Finally, antibodies against β-arrestin-1 and 2 reveal 4 immunoreactive species in the 48-52 kD range. Thus, the microvillar membrane preparation we developed houses the transduction machinery for activation and desensitization of vomeronasal neurons in response to pheromones.

Our studies extend those by Halpern *et al.* (1995) in showing that vomeronasal neurons express at least five different G proteins with distinct expression patterns. The microvillar membrane preparation, which we developed, is likely to facilitate future studies to assign specific functions to each of these vomeronasal G proteins. Based on our observations, it is likely that in the dendritic microvilli of vomeronasal neurons G_s is coupled to adenylate cyclase and the G_q-related protein to phospholipase C (Taylor *et al.*, 1991).

Finally, we have extracted mRNA from vomeronasal organs of gilts and constructed a high titer cDNA expression library in Lambda ZAP Express with average insert sizes of 1.4 kb. We are currently using sequence information from Dulac and Axel (1995) to amplify genomic DNA sequences from mouse DNA that correspond to putative pheromone receptor sequences. These PCR products will then be used to screen our porcine cDNA library to identify cDNAs encoding putative porcine pheromone receptors. Such cDNAs can be expressed in transfected cells and fractions of boar urine can be tested for their ability to generate inositol-(1,4,5)-triphosphate via putative pheromone receptors. These studies will lead both to the functional characterization of pheromone receptors and the chemical identification of urine-born pheromones. Support for the continuation of these studies, which we could initiate thanks to support from ARO grant DAAH04-94-G-0027, has been requested from the North Carolina Biotechnology Center and the NIH.

C. LIST OF ALL PUBLICATIONS AND TECHNICAL REPORTS

1. Journal articles

1. Anholt, R. R. H., Lyman, R. F. and Mackay, T. F. C. (1996) Effects of single *P*-element insertions on olfactory behavior in *Drosophila melanogaster*. Genetics, in press.

2. Anholt, R. R. H., Carlson, M. R., Wekesa, K. S., Kwok, Y. L. and Vandenbergh, J. G. (1996) Chemosensory transduction enzymes and G proteins in microvillar membranes from mammalian vomeronasal organ. J. Neurosci., submitted.

3. Mackay, T. F. C., Hackett, J. B., Lyman, R. F., Wayne, M. L. and Anholt, R. R. H. (1996) Quantitative genetic variation of odor-guided behavior in a natural population of *Drosophila melanogaster*. Genetics, in preparation.

2. Invited reviews

1. Anholt, R. R. H. (1993) Molecular neurobiology of olfaction. Critical Rev. Neurobiol. **7**: 1-22.

2. Anholt, R. R. H. (1994) Signal integration in the nervous system: Adenylate cyclases as molecular coincidence detectors. Trends Neurosci., **17**: 37-41.

3. Book chapters

1. Menini, A. and Anholt, R. R. H. (1994) Cyclic nucleotide-activated channels. In: Endocrinology and Metabolism. Progress in Research and Clinical Practice. Vol. 6. Ion Channels and Ion Pumps: Metabolic and Endocrine Relationships in Biology and Clinical Medicine (Editors, P. P. Foa and M. F. Walsh), Chapter 24, pp. 526-548, Springer Verlag, New York, NY.

2. Anholt, R. R. H. (1995) Preparation of olfactory cilia. In: Experimental Cell Biology of Taste and Olfaction: Current Techniques and Protocols (Editors A. I. Spielman and J. G. Brand), CRC Press, Inc., Boca Raton, FL., in press.

4. Abstracts

1. Anholt, R. R. H., Lyman, R. F. and Mackay, T. F. C. (1994) Identification of olfactory genes by single *P*-element mutagenesis of inbred *Drosophila melanogaster*. Sixteenth Annual Meeting of the Association for Chemoreception Sciences, Abst. 224.

2. Lyman, R. F., Mackay, T. F. C., and Anholt, R. R. H. (1994) Olfactory genes identified from a behavioral screen of *Drosophila melanogaster* lines carrying single *P*-element inserts. Eleventh Congress of the European Chemoreception Research Organization (Blois, France), Abst. 74.

3. Anholt, R., Lyman, R. and Mackay, T. (1995) Quantitative trait loci for olfaction in *Drosophila melanogaster*. Thirty-sixth Annual Drosophila Research Conference (Atlanta, GA), p. 38.

4. Anholt, R. R. H. (1995) Olfaction and reproduction: Molecular aspects of odor recognition and signal transduction. 1995 Triangle Conference on Reproductive Biology, Abst. #3.

5. Anholt, R. R. H., Carlson, M. A., Kwok, Y. L., Oppermann, M., Wekesa, K. S., Lefkowitz, R. J. and Vandenbergh, J. G. (1996) Characterization of transduction enzymes and G proteins in microvillar membrane preparations from mammalian vomeronasal organ. Eighteenth Annual Meeting of the Association for Chemoreception Sciences, in press.

6. Kulkarni, N. H., Nuzhdin, S. V., Mackay, T. F. C. and Anholt, R. R. H. (1996) Characterization of *smell impaired* genes of *Drosophila melanogaster*. Eighteenth Annual Meeting of the Association for Chemoreception Sciences, in press.

7. Anholt, R. R. H., Carlson, M. R., Wekesa, K. S., Kwok, Y. L. and Vandenbergh, J. G. (1996) Chemosensory transduction enzymes and G proteins in microvillar membranes from mammalian vomeronasal organ. 1996 Triangle Conference on Reproductive Biology, Abst. #20.

D. LIST OF ALL PARTICIPATING SCIENTIFIC PERSONNEL

The Principal Investigator, Dr. Robert R. H. Anholt devoted 50% effort to ARO-sponsored research. Ms. Yee-Lut Kwok, a Research Technician II, was assigned full-time to research sponsored by ARO. Three undergraduate students, Tricia Henson, Marie Carlson and Brant Hackett, also participated in the research described in this report. In addition, we have greatly benefitted from collaborative interactions with the laboratories of Drs. T. F. C. Mackay (Genetics, NCSU), J. G. Vandenbergh (Zoology, NCSU), and R. J. Lefkowitz (Howard Hughes Medical Institute, Duke Univ.).

5. REPORT OF INVENTIONS

1. Anholt, R. R. H. and Mackay, T. F. C. (1994) Use of pyrazines and thiazoles as fly repellents. Patent disclosure NCSU 95-2.

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7. APPENDICES

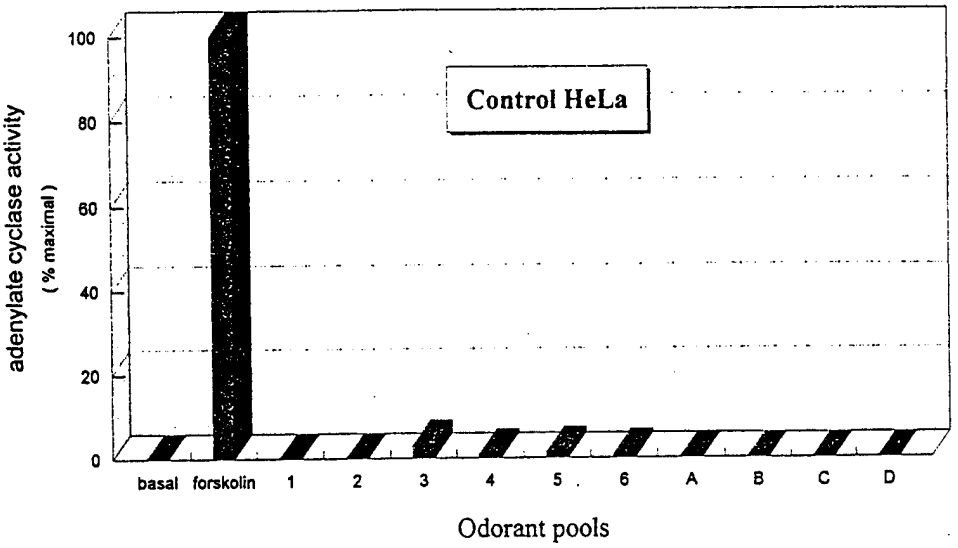
Figure 1: Activation of adenylyl cyclase in control Hela cells and transfected HeLa cells expressing a chimeric β_2-adrenergic receptor containing the hypervariable odorant binding domain of rat odorant receptor I-15 by pools of odorants.

Figure 2: Generation of inositol-(1,4,5)-triphosphate by boar urine in microvillar membranes from vomeronasal organs of 3 months-old prepubertal gilts.

Table 1: Odorants used for initial screening assays.

Table 2: *P[lArB]* insert lines of *Drosophila melanogaster* with aberrant olfactory behavior.

Table 3: Generation of cyclic AMP and inositol-(1,4,5)-triphosphate in microvillar membrane preparations from female porcine vomeronasal organs.



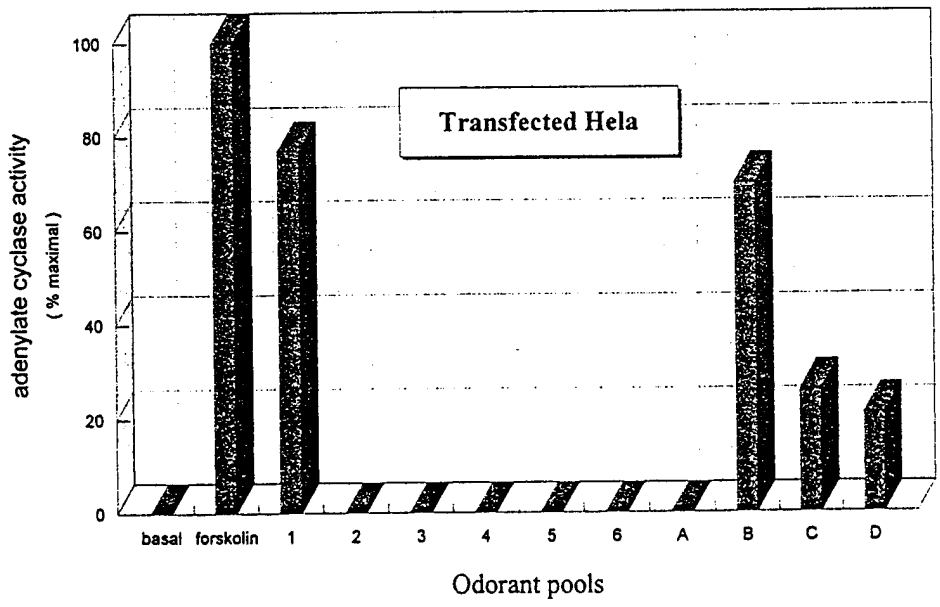


Figure 1: Activation of adenylyl cyclase in control Hela cells (upper panel) and transfected HeLa cells expressing a chimeric β_2-adrenergic receptor containing the hypervariable odorant binding domain of rat odorant receptor I-15 (lower panel) by pools of odorants from Group 1 (Table 1). Activity is expressed as percentage of forskolin-stimulated activity. Significant stimulation by odorants is observed only in transfected cells and only with odorant pools 1 and B. The common odorant in these pools is furfuryl propionate.

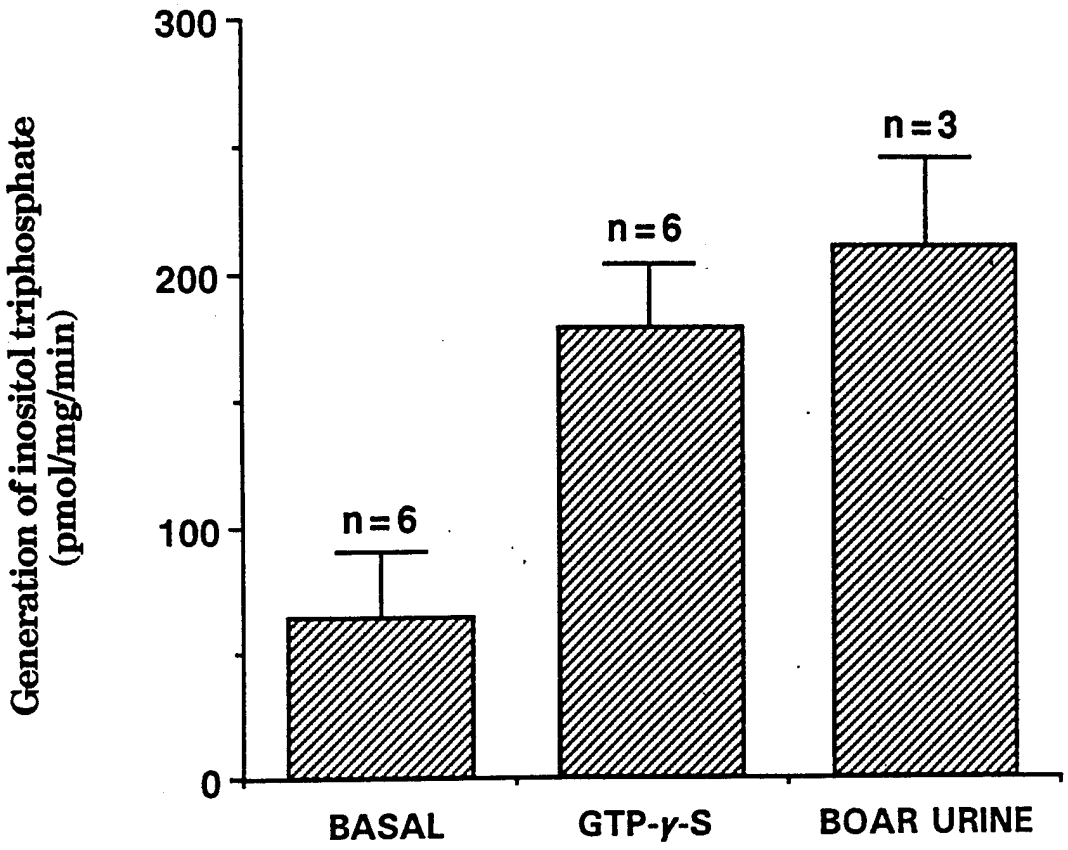


Figure 2: Generation of inositol-(1,4,5)-triphosphate by boar urine in microvillar membranes from vomeronasal organs of 3 month-old prepubertal gilts. Reactions were performed in the absence of stimulus, in the presence of 10 μM GTPγS, or in the presence of 10% (v/v) boar urine. All assays were done in the presence of 10 μM GTP. Significant stimulation compared to the basal activity is observed in the presence of GTPγS or boar urine ($p < 0.02$ by two-tailed Student's *t*-test). Stimulation of inositol-(1,4,5)-triphosphate production by GTPγS is not statistically different from stimulation by boar urine.

TABLE 1: ODORANTS USED FOR INITIAL SCREENING ASSAYS

GROUP 1: ESTERS

	A.	B.	C.	D.
I.	ethyl propionate	furfuryl propionate	methyl butyrate	methyl tiglate
II.	ethyl butyrate	furfuryl butyrate	propyl butyrate	propyl tiglate
III.	ethyl pentanoate	furfuryl pentanoate	isopropyl butyrate	isopropyl tiglate
IV.	ethyl hexanoate	furfuryl hexanoate	hexyl butyrate	hexyl tiglate
V.	ethyl heptanoate	furfuryl heptanoate	allyl butyrate	allyl tiglate
VI.	ethyl octanoate	furfuryl octanoate	benzyl butyrate	benzyl tiglate

GROUP 2: HETEROCYCLICS

	A.	B.	C.	D.
I.	2-acetylpyrrole	thiazole	2-methylpyrazine	2-ethylpyrazine
II.	2-acetylfuran	4-methylthiazole	2,3-dimethylpyrazine	2,3-diethylpyrazine
III.	2-acetylthiophene	4,5-dimethylthiazole	2,5-dimethylpyrazine	3-ethyl-2-methylpyrazine
IV.	2-acetylthiazole	2,4,5-trimethylthiazole	2,6-dimethylpyrazine	2-methoxypyrazine
V.	2-acetylpyridine	2-isobutylthiazole	2,3,5-trimethylpyrazine	2-methoxy,3-methylpyrazine
VI.	2-acetylpyrazine	2-ethoxythiazole	2,3,5,6-tetramethylpyrazine	2-isobutyl,3-methoxypyrazine

GROUP 3: TRADITIONAL EXPERIMENTAL ODORANTS

	A.	B.	C.	D.
I.	citralva	acetophenone	l-carvone	triethylamine
II.	citronellal	lilial	d-carvone	phenylethylamine
III.	limonene	α-pinene	menthone	isobutyric acid
IV.	lyral	phenyl ethyl alcohol	cineole	isovaleric acid
V.	β-ionone	geraniol	eugenol	ethyl vanillin
VI.	isoamyl acetate	amylsalicylate	cinnamic aldehyde	benzaldehyde

Rows, labeled I-VI, and columns, labeled A-D, represent pools of odorants. Within the 10 pools of each group each odorant is represented twice. For initial assays each odorant is present at a final concentration of 1 μM.

Table 2: *P[lArB]* insert lines of *Drosophila melanogaster* with aberrant olfactory behavior.

line	avoidance score (average ± S.E.M. (n; *t*)) benzaldehyde 100%	benzaldehyde 1%	2-isobutylthiazole	2-*n*-propylpyrazine	*lacZ* expression pattern
***Sam ry^{506}* control**	**3.98 ± 0.04 (90;-)**	**3.65 ± 0.08 (87;-)**	**3.66 ± 0.08 (97;-)**	**3.58 ± 0.09 (55;-)**	
			second chromosome lines		
smi21F	4.12 ± 0.13 (18; *-1.10*^{ns})	♂: 3.76 ± 0.27 (9; *-0.38*^{ns}) ♀: 2.51 ± 0.26 (9; *4.43***)	3.60 ± 0.14 (24; *0.42*^{ns}) 3.05 ± 0.14 (21; *4.36****)	3.38 ± 0.21 (21; *0.95*^{ns}) 3.29 ± 0.19 (21; *1.54*^{ns})	heavy punctate staining in proximal region of 3rd antennal segment; faint staining in maxillary palps.
smi26D	2.94 ± 0.14 (10; *5.05****)	2.60 ± 0.26 (18; *4.02****)	2.11 ± 0.19 (24; *8.18****)	2.24 ± 0.30 (12; *4.44****)	no staining detected
smi27E	3.00 ± 0.14 (10; *4.18***)	2.23 ± 0.17 (12; *8.35****)	2.19 ± 0.17 (24; *8.76****)	2.38 ± 0.36 (12; *3.37***)	3rd antennal segment (faint)
smi28E	2.82 ± 0.17 (10; *6.78****)	2.54 ± 0.26(12; *4.34***)	2.35 ± 0.24 (17; *5.50****)	2.30 ± 0.30 (12; *4.29***)	punctate staining throughout 3rd antennal segment
smi35A	3.66 ± 0.18 (18; *1.80**)	3.19 ± 0.15 (30; 2.99**)	3.23 ± 0.15 (30; *2.86***)	3.05 ± 0.20 (24; *2.75***)	3rd antennal segment (faint)
smi45E ♂	4.22 ± 0.22 (9;*-1.11*^{ns})	3.68 ± 0.20 (12; *-0.15*^{ns})	3.60 ± 0.14 (24; *0.47*^{ns})	2.94 ± 0.22 (18; *2.89***)	2nd and 3rd antennal segments; maxillary palps.
♀	3.12 ± 0.32 (9; *2.68**)	2.63 ± 0.33 (12; *3.14***)	3.08 ± 0.20 (24; *2.94***)	2.73 ± 0.24 (18; *3.52***)	
smi51A ♂	4.05 ± 0.11 (12; *-0.64*^{ns})	3.80± 0.23 (9; *-0.63*^{ns})	3.70 ± 0.15 (30; *-0.27*^{ns})	3.22 ± 0.20 (12; *1.86**)	2nd and 3rd antennal segment.
♀	2.86 ± 0.21 (12; *5.33****)	2.64 ± 0.32 (9; *3.15***)	3.29 ± 0.16 (30; *2.36**)	2.28 ± 0.41 (12; *3.22***)	
smi60E	2.67 ± 0.29 (10; *4.50***)	2.27 ± 0.33 (11; *4.22***)	2.22 ± 0.20 (24; *7.10****)	2.45 ± 0.28 (12; *4.04***)	3rd antennal segment
			third chromosome lines		
smi61A	4.06 ± 0.14 (18; *-0.58*^{ns})	3.21 ± 0.18 (30; *2.42**)	3.14 ± 0.23 (18; *2.29**)	3.17 ± 0.15 (24; *2.72***)	2nd and 3rd antennal segment
smi65A	3.26 ± 0.30 (8; *2.41**)	2.93 ± 0.27 (12; *2.70**)	2.97 ± 0.21 (18; *3.22***)	2.39 ± 0.16 (12; *7.36****)	no staining detected
smi79E	2.93 ± 0.30 (12; *3.50***)	2.63 ± 0.25 (11; *4.07***)	3.19 ± 0.23 (18; *2.00**)	2.90 ± 0.26 (11; *2.61**)	no staining detected
smi97B ♂	3.44 ± 0.21 (9; *2.50**)	2.52 ± 0.40 (9; *2.80**)	1.81 ± 0.15 (16; *12.13****)	1.96 ± 0.26 (12; *6.38****)	localized staining in 3rd antennal segment near base of arista.
♀	2.31 ± 0.18 (9; *9.48****)	1.43 ± 0.24 (8; *9.17****)	1.62 ± 0.22 (18; *9.30****)	1.19 ± 0.21 (12; *11.28****)	
smi98B	3.32 ± 0.19 (18; *3.55***)	2.87 ± 0.20 (12; *3.91***)	3.11 ± 0.21 (18; *2.66***)	1.90 ± 0.16 (18; *10.33****)	scattered cells in 3rd antennal segment
P(3)217	3.32 ± 0.20 (24; *3.34***)	1.83 ± 0.25 (11; *7.39****)	3.04 ± 0.26 (18; *2.41**)	2.11 ± 0.23 (18; *6.38****)	no staining detected

Avoidance scores of *P*-element insert lines were compared to those of *P*-element free *Sam ry^{506}* controls by one-tailed Student's *t*-test. ***: $p < 0.001$; ** : $0.001 < p < 0.01$; *: $0.01 < p < 0.05$; ^{ns}: $p > 0.05$.

When tested at 100% benzaldehyde, lines *smi45E, smi51A* and *smi97B* show significant sexual dimorphism ($p < 0.01$). When tested at 1% benzaldehyde, sexual dimorphism is observed in lines *smi21F* ($p < 0.01$), *smi45E* ($p < 0.02$), *smi51A* ($p < 0.02$) and *smi97B* ($p < 0.05$). When tested with 2-isobutylthiazole sexual dimorphism is observed in lines *smi21F* ($p < 0.05$), *smi45E* ($p < 0.01$) and *smi51A* ($p < 0.02$). When tested with 2-*n*-propylpyrazine, sexual dimorphism is observed in lines *smi51A* ($p < 0.05$) and *smi97B* ($p < 0.05$). In each case the statistical significance of sexual dimorphism was evaluated by two-tailed Student's *t*-test.

The avoidance score to distilled water was 1.76 ± 0.06 (n = 170).

Table 3: Generation of cyclic AMP and inositol-(1,4,5)-triphosphate in microvillar membrane preparations from female porcine vomeronasal organs

membrane fraction	yield of membranes µg protein/organ ± S.E.M. (*n*)	distribution of membrane protein in the fractions (percent of total)	adenylate cyclase activity (pmol/min/mg protein) av. ± S.E.M. (*n*)			generation of inositol triphosphate (pmol/min/mg protein) av. ± S.E.M. (*n*)	
			basal	GTPγS-stimulated	forskolin-stimulated	basal	GTPγS-stimulated
1. RESIDUAL	4,145 ± 452 (*11*)	96.7	32 ± 5 (*5*)	53 ± 7 (*5*)	76 ± 10 (*5*)	29 ± 17 (*6*)	54 ± 9 (*6*)
2. MICRO-VILLAR	141 ± 9 (*11*)	3.3	68 ± 12 (*5*)	117 ± 13 (*5*)	228 ± 38 (*5*)	64 ± 26 (*6*)	178 ± 26 (*6*)
			(enrichment of forskolin-stimulated activity in the microvillar fraction: app. 3.0-fold)			(enrichment of GTPγS-stimulated activity in the microvillar fraction: app. 3.3-fold)	